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Neurotoxicity, and Role in Gulf War Illness

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13. ABSTRACT (Maximum 200 Words) This proposal investigates the potential for inhaled uranium oxide (UO) aerosols to penetrate the nose-brain barrier, directly enter the central nervous system (CNS), diffusely distribute within the CNS, and result in slowly developing neurotoxic responses. Potentially substantial inhalation exposures to depleted uranium (DU) occurred during the GW and recent data suggests systemic DU enters the CNS and is associated with neurological deficits. Penetration of the nose-brain barrier can produce deposition of metals in the CNS that swamps concentrations seen in target organs of systemic deposition. Because several conditions during the war could have produced nasal inflammation, inflammation will be examined as a modifying factor that could result in increased sensitivity to uranium uptake via penetration of the nose-brain barrier. Nephrotoxic and pulmonary effects will be evaluated to determine whether CNS effects can occur at lower thresholds than nephrotoxic effects. To date, data have been analyzed from animals exposed to high concentration (~500 mg/m ³) 15 minute exposures to uranium oxides varying in solubility, with or without prior endotoxin-induced inflammation. Under these conditions, female rats died of kidney toxicity within 2 weeks postexposure. Although uptake of uranium was generally not observed within the CNS, exposures were sufficient to produce neuroinflammation, with degree of inflammation positively associated with the solubility of the uranium oxide.				
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**Inhalation of Uranium Oxide Aerosols: CNS Deposition, Neurotoxicity and Role in Gulf
War Illness -
Annual Report: September 2003**

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INTRODUCTION

Purpose: The purpose of the overall project is to test the hypothesis that inhaled uranium-containing aerosols will enter the central nervous system (CNS) via olfactory transport, follow neuronal pathways to distal regions of the CNS, and ultimately result in neurodegeneration. The studies in Year 2 address metal uptake and neuroinflammation after short-term, high concentration exposures or long term, low concentration exposures to uranium oxides and depleted uranium oxide. These studies examine the effects of inhaled uranium oxides both in a healthy rat model and one in which inflammation has been induced in the upper respiratory tract. Target organs to be examined include nasal and lung tissue, brain, and kidney. A one-year time course of response following the exposures will be determined with sacrifices scheduled on 0, 30, 180, and 360 days post-exposure. Results of these exposures will address all of the original hypotheses:

Hypothesis I. Inhalation of uranium aerosols during the Gulf War from combustion of DU containing weapons resulted in CNS deposition and subsequent neurodegeneration in a subset of those exposed.

Hypothesis II: Transient conditions such as inflammation compromised the olfactory epithelium and enhance the entry of uranium and the subsequent development of neurodegeneration

Hypothesis III: Markers of neurodegeneration are correlated with the concentration and deposition of U within the CNS following inhalation exposure.

Hypothesis IV: The degree of and time-course of neurodegeneration are dose and exposure duration dependent.

These hypotheses will be tested for both short- and long-term exposure scenarios as differences related to exposure rate and dose may be critical to uptake, clearance, and ultimate neurotoxicity.

BODY

Approved Scope of Work for Year 2

Year 1 Scope as a prelude to Year 2 tasks

During Year 1, rats will be exposed via nose-only inhalation for 15 minutes to aerosols of 1) insoluble UO_2 ; 2) soluble UO_3 ; 3) a mixture containing by weight 50% UO_2 and 50% UO_3 ; 4) TaO_2 (a negative control) at concentrations of 500 mg/m^3 . A fifth group will be co-exposed to endotoxin (to induce inflammation) and the $\text{UO}_2 + \text{UO}_3$ mixture for 15 minutes. Thirty animals will be exposed in each group. Separate air-only and endotoxin-air exposed control groups will be employed as vehicle controls. For each group rats will be sacrificed in sets of 6 per group immediately following exposure, and at 30, 180 and 360 days post-exposure. Tissue analyses from the short-term exposures will begin in Year 1, but substantially carry over to Year 2.

Year 2 Scope

Quantitation of metals in nose and brain tissues will be performed with Atomic Absorption Spectrophotometry (AAS) and microbeam Proton Induced X-ray Emission (μ -PIXE). Immunohistochemistry (IHC) of heat shock proteins (HSP) will be done on nasal and representative brain tissues. Brain tissues will also be examined using IHC for the persistence of tyrosine hydroxylase - containing neurons and for the persistence of NeuN labelling. Neuroinflammation will be monitored by GFAP (glial fibrillary acidic protein) -IHC. A marker of neuronal degeneration (Fluor Jade histochemistry) and a preliminary indicator of the neuronal apoptosis (TUNEL) will also be examined in sections from brains showing U deposition. If data from any of these categories indicates that there is neurodegeneration, specific brain areas such as the substantia nigra (SN) and anatomically-linked areas will be studied in greater detail using IHC directed against other DA cell markers such as the postsynaptic receptors D1 and D2 within the substantia nigra, caudate putamen and olfactory bulbs. Immunoinflammatory markers (IL-1, IL-6, TNF α and their receptors) and immunohistochemistry to detect 4-hydroxynonelation of nigral dopaminergic neurons will also be performed.

One kidney and one lung from each sacrificed animal will be analyzed by AAS for inhaled metal content. The other lung and kidney from each sacrificed animal will undergo histopathological examination. Data from the kidney analyses will allow us a measure of potential nephrotoxicity and will afford a comparison of inhalation data with nephrotoxic effects arising from the study of DU implants. Data from lung will afford an assessment of any pulmonary damage resulting from the inhalation exposures.

Multivariate analyses will be used to examine differences between the groups in both the concentrations of metal localized within different brain regions, and the levels of the indicators of neurodegeneration and other markers analyzed. In conditions where the N is not sufficient for this parametric analysis, nonparametric and qualitative analyses will be utilized instead.

Longer duration exposures will be performed in year 2 and 3. Serial sacrifices of rats exposed via nose-only inhalation for 8 hours to aerosols of a mixture containing by weight 50% UO₂ and 50% UO₃ at a dose of 1 mg/m³. A separate air-only exposed control group will be employed as vehicle control. For each group rats will be sacrificed in sets of 6 per group immediately following exposure, and at 30, 180 and 360 days post-exposure. Tissue analyses will begin in Year 2, but substantially carry over to Year 3.

Progress on Year Two Scope

The following section is organized by task identified in the original scope of work documented above. The relevant section of the scope, quoted and in bold italics, begins each description of work.

TASK 1: *Rats will be exposed via nose-only inhalation for 15 minutes to aerosols of 1) insoluble UO₂; 2) soluble UO₃; 3) a mixture containing by weight 50% UO₂ and 50% UO₃; 4) TaO₂ For each group rats will be sacrificed in sets of 6 per group immediately following exposure, and at 30, 180 and 360 days post-exposure.*

Methods Task 1: Short-term exposure sacrifices

Exposures and sacrifice of 0 and 30 day survival times was completed in Year 1. The 180 and 360 day survival groups were sacrificed in Year 2. Rats were sacrificed by exposure to CO₂ and exsanguination by cardiac saline perfusion. For rats in the 180 day groups, brains were removed and frozen in liquid-nitrogen-cooled isopentane at -36°C and transferred at the end of the day to -80°C for long term storage. For rats in the 360 day groups, saline perfusion was immediately followed by perfusion with 4% paraformaldehyde. Brains were removed and post-fixed in 4% paraformaldehyde for 2 h before being transferred to 10% sucrose solution for storage.

The nose, with skin and lower jaw removed was fixed in 4% paraformaldehyde. The left and right lungs are weighed. The left lung was perfused with 4% paraformaldehyde and the right was frozen. The larynx, trachea and bronchial lymph node were fixed in 4% paraformaldehyde. The left and right kidneys were weighed. The left kidney was fixed in 4% paraformaldehyde for histopathology and the right was frozen for subsequent uranium analysis. Both femurs were weighed and frozen for chemical analysis.

Results Task 1: Short-term exposure sacrifices

All animals subjected to 15-min short-term exposures have been sacrificed (Tables 1 and 2). Sacrifices of rats 180 and 360 days post-exposure were performed during Year 2. The concentration and particle size of uranium aerosols for short-term exposures are included in Table 3 for clarification.

Table 1. Short-term exposure: uranium entry to CNS

Material	Number of animals (male/female)				Date of sacrifice			
	0 d	30 d	180 d	360 d	0 d	30 d	180 d	360 d
Air**	2m/2f	2m/2f	2m/2f	4m/4f	8/5/02	9/4/02	2/4-6/03	8/4-8/03
UO ₂	3m/3f	3m/3f	3m/3f	7m/7f	8/8/02	9/5/02	2/4-6/03	8/4-8/03
UO ₃	3m/3f	3m/1f	3m/0f	4m/0f	8/6/02	9/3/02	2/4-6/03	8/4-8/03
UO ₂ +UO ₃	3m/3f	3m/3f	3m/3f	7m/7f	8/9/02	9/6/02	2/4-6/03	8/4-8/03
TaO ₂	3m/3f	3m/3f	3m/3f	6m/7f	8/5/02	9/3/02	2/4-6/03	8/4-8/03
DUO _x **	3m/3f	3m/3f	3m/3f	7m/7f	8/7/02	9/4/02	2/4-6/03	8/4-8/03
Total # rats	34	32	31	67				

Table 2. Short-term exposure: inflammation and uranium entry to CNS

Material	Number of animals (male/female)				Date of sacrifice			
	0 d	30 d	180 d	360 d	0 d	30 d	180 d	360 d
Air	2m/2f	2m/2f	2m/2f	4m/4f	8/6/02	9/3/02	2/4/03	8/4-8/03
Air + Endotoxin	3m/3f	3m/3f	3m/3f	6m/5f	8/8/02	9/5/02	2/4-6/03	8/4-8/03
UO ₂ +UO ₃	Same as UO ₂ +UO ₃ group in Table 1							
UO ₂ +UO ₃ + Endotoxin	3m/3f	3m/3f	3m/3f	6m/6f	8/9/02	9/6/02	2/4-6/03	8/4-8/03
DUOx	Same as DUOx group in Table 1							
DUO _x + Endotoxin	3m/3f	3m/3f	3m/3f	6m/4f	8/7/02	9/4/02	2/4-6/03	8/4-8/03
Total # rats	22	22	22	41				

Table 3. Aerosols used for short-term exposures

CHEMICAL COMPOUND	MEAN CONCENTRATION (mg/m ³)	PARTICLE SIZE (MMAD)	Sigma-g
TaO ₅	548.01	2.13	1.92
UO ₃	328.75	1.56	1.71
DU	608.81	2.02	1.36
UO ₂	572.05	2.38	1.39
UO ₂ + UO ₃	304.66	1.97	1.52

TASK 2: *Quantitation of metals in nose and brain tissues will be performed with Atomic Absorption Spectrophotometry (AAS) and microbeam Proton Induced X-ray Emission (μ -PIXE)*

Methods Task 2: PIXE analysis of brains from short-term exposures

Tissue sectioning and preparation

Frozen tissue sections were cut at 10 μ m using a Hacker-Bright motor-driven cryostat. Three levels of sagittal sections containing the brain regions of interest were sampled. These areas were chosen to allow analysis of the primary projection regions of receptor neurons located within the nose (olfactory glomeruli and the spinal nucleus of the trigeminal), and projection regions for the olfactory system and nuclei within the dopaminergic system. One section was mounted on a nylon foil for PIXE analysis. Eight sections from the same level were mounted on Fisher ProbeOn Plus glass slides and retained for immunohistochemical analysis. Anatomical regions within each of the levels are summarized in Table 4. Serial sections of those sent to LLNL for PIXE analysis were stained with hematoxylin and eosin, digitally scanned (Figure 1A), and regions for PIXE analysis were outlined to ensure beam localization within correct brain regions (Figure 1B).

Table 4. Brain regions within each sampled sagittal level of rat brain

Sectioning level	Anatomic Structure	Lateral distance from midline	Number of sections
1	Caudate putamen Spinal nucleus	~2.9 mm	6-8
2	Substantia nigra Spinal nucleus	~1.9 mm	6-8
3	Olfactory bulb Substantia nigra	~0.9 mm	6-8

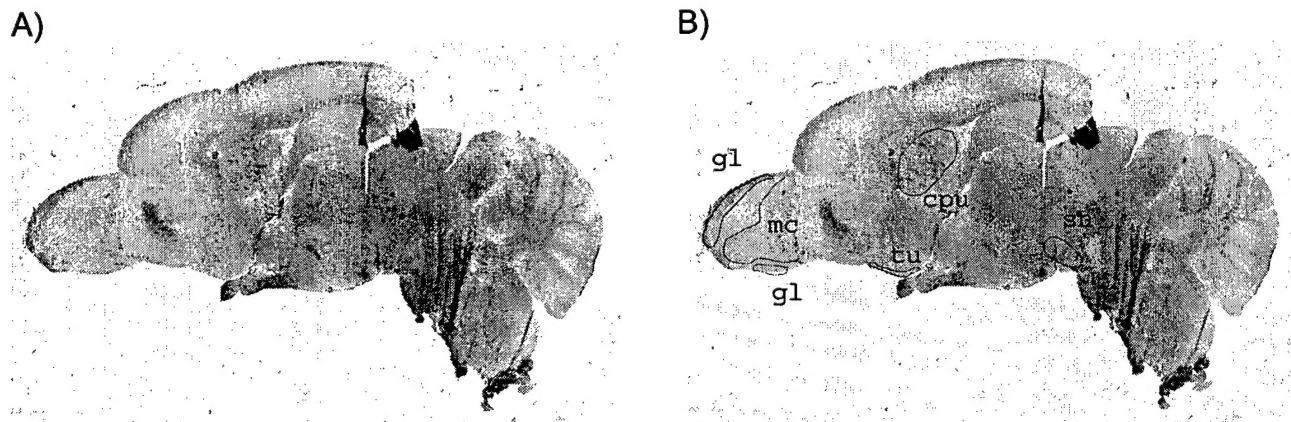


Figure 1. A) Digitally scanned photomicrograph of serial sagittal brain section sent for PIXE analysis. B) Serial sagittal brain section with anatomic structures of interest outlined where: gl=glomeruli; mc=mitral cells; tu=tuberculum; cpu=caudate putamen; sn=substantia nigra.

PIXE measurement of metal content

Uranium and tantalum (Ta) concentrations in localized brain regions were determined with Proton Induced X-ray Emission (PIXE). PIXE is an x-ray fluorescence technique that uses MeV energy proton beams to interrogate specimens. It provides accurate quantitation, simultaneous multi-element detection and is capable of micron-scale spatial resolution whilst maintaining down to 1 mg/g elemental sensitivity. Regions of interest within the freeze-dried tissue sections were identified visually using stained adjacent serial sections. Regions of interest were irradiated with 3 MeV proton microbeams for doses of up to 15 micro coulombs. Beam spot sizes were typically between 0.3x0.3 and 0.5x0.5 mm. X-ray yields for U and Ta were monitored using an energy dispersive x-ray detector. Yields were converted to quantitative concentrations using thin film standards of Ta and U of known thickness to determine detector efficiency. The system has been tested on certified standards and has quantitative accuracy of better than 95% for analysis of metals in biological matrices.

Results Task 2: PIXE analysis of brains from short-term exposures

Uranium content in glomeruli and mitral cells from the animals sacrificed at day of exposure (day 0) was analyzed in Year 1. During Year 2, we have extended the PIXE analysis to also

include brains from all animals sacrificed 30 days after exposure. In addition additional brain structures (olfactory tubercule, spinal nucleus, substantia nigra and caudate putamen) were evaluated in all groups for both timepoints.

Uranium levels were below the detectable level (MDL) of 2.4-2.7 µg/mg (Table 5) for all groups and brains structures at the 0 day sacrifice. These animals were actually sacrificed at 2 hrs post exposure on that day. One animal in the most soluble uranium exposure condition, UO₃, had detectable uranium (3.8 µg/mg) in the mitral cell layer of the olfactory bulb at 30 days post exposure, and one animal in the UO₂ exposure condition showed detectable uranium (4.1 µg/mg) at 30 days post exposure within the olfactory tuberculum (Table 6). Due to the low frequency of detection and low concentrations detected at 30 days post-exposure, later time-points (180 and 360 days) were not analyzed for uranium content. Results from previous analyses with other metals indicated that the 30 day results would predict no detectable uranium in brain structures at 180 days.

Table 5. Characterization of MDLs across Brain Regions Analyzed.
95% CI

Structure	Average MDL	SE	Lower	Upper
CPU	2.53	0.05	2.43	2.63
Glomeruli	2.60	0.03	2.53	2.66
Mitral	2.62	0.03	2.56	2.69
SN	2.55	0.05	2.45	2.65
SP	2.67	0.05	2.58	2.76
TU	2.43	0.05	2.33	2.53
Overall	2.58	0.02	2.54	2.62

Table 6. Number of PIXE uranium measurements in different brain structures

	Glomeruli	Mitral cells	Spinal Nucleus	Caudate Putamen	Substantia Nigra	Tubercule
Exposure	15	15	6	7	6	7
Air	12	12	6	6	6	6
DUOx	12	12	6	6	6	6
DUOx + Endotoxin	12	12	6	6	6	6
Endotoxin	3	3	4	3	4	3
TaO2	12	12	6	6	6	6*
UO2	11	11	5	5	6	5
UO2 +UO3	10	10**	7	4	4	3
UO3	11	11	3	6	5	4
UO2 +UO3 + endotoxin	15	15	6	7	6	7

Except for 2 measurements all are less than minimum detection limits. * single measured value = 3.8 mg/kg, ** single measured value = 4.1 mg/kg

TASK 3: *Tissue analyses from the short-term exposures will begin in Year 1, but substantially carry over to Year 2. Neuroinflammation will be monitored by GFAP (glial fibrillary acidic protein) -IHC*

Methods Task 3: Neuroinflammation in brains from short-term exposures

Note: Year 1's progress report contained preliminary analyses on GFAP immunoreactivity. The full dataset is now analyzed for 0 and 30 day sacrifice times. The full dataset will be reported in this section, but the comparison with last year's preliminary results will be discussed in the conclusions section. Data reported here include restaining of sections from the animals included in last year's result rather than inclusion of those stained sections.

Technical Difficulties

The microtome and drive mechanisms in the cryostat used to microsection brains in these studies became unreliable during early 2003. Repair technicians locally exacerbated the problem, resulting in the need to ship the machine to California for extensive repairs. Although efforts were made to continue through borrowing time on other machines at the Health Sciences Center, work was slowed due to this loss of equipment. The repaired cryostat was returned to Albuquerque in October, and work is again progressing.

GFAP immunohistochemistry

Sections from level 3 (see above for methods, Task 2) containing the olfactory bulb were reacted with antibody to GFAP (Rabbit anti-cow GFAP-[DAKO Corporation, Denmark]) at a 1:500 dilution. Cy3-conjugated AffiniPure donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at a 1:400 dilution was used as the secondary fluorescent-tagged antibody. The staining protocol was performed using FisherBiotech Microprobe Manual Staining System which utilizes capillary gap technology to ensure complete and uniform coverage of tissue with reagents. All slides were randomized into batches of 20 and included air and uranium exposed tissues, as well as control (non-exposed standard) tissues to allow assessment of any batch effects across all exposure groups. All tissues were stained within a 1 day period to also minimize any staining differential associated with procedural variance.

Analysis of GFAP staining intensity

Density of GFAP in stained sections was determined from digital micrographs of the fluorescent images. Exposure time was kept constant between sections. Photographs of hematoxylin and eosin stained sections were also taken to ensure correct glomerular location (see Figures 2 A and B).

The sampling and analysis procedure is graphically summarized in Figure 3. Sections for analysis were cut from images using a standard 150 x 150 pixel ellipse mask in Adobe Photoshop (Version 7.0) software. These cut samples were then transferred to ImageTool (Version 5, UTHSCSA) software for densitometric analysis. Ellipse samples were pasted within a new image stack to undergo threshold processing to convert the grayscale image

into true black and white. The threshold window for all slides was adjusted to enable visualization of neurons and dendrites while still controlling for any background staining. This threshold was kept constant across all tissues to maintain consistency across slides and batches (see Figures 2 C and D). The number and percent of black and white pixels was then tabulated via an ImageTool analysis function. Three separate sections were cut from each image and analyzed to calculate a mean value for number and percent black pixels, the indicator for total amount of electrofluorescence staining.

The three black density values for each image were then averaged to compute a mean density value for that animal. Mean values for male, female, and combined group were determined for density and percent black in the image.

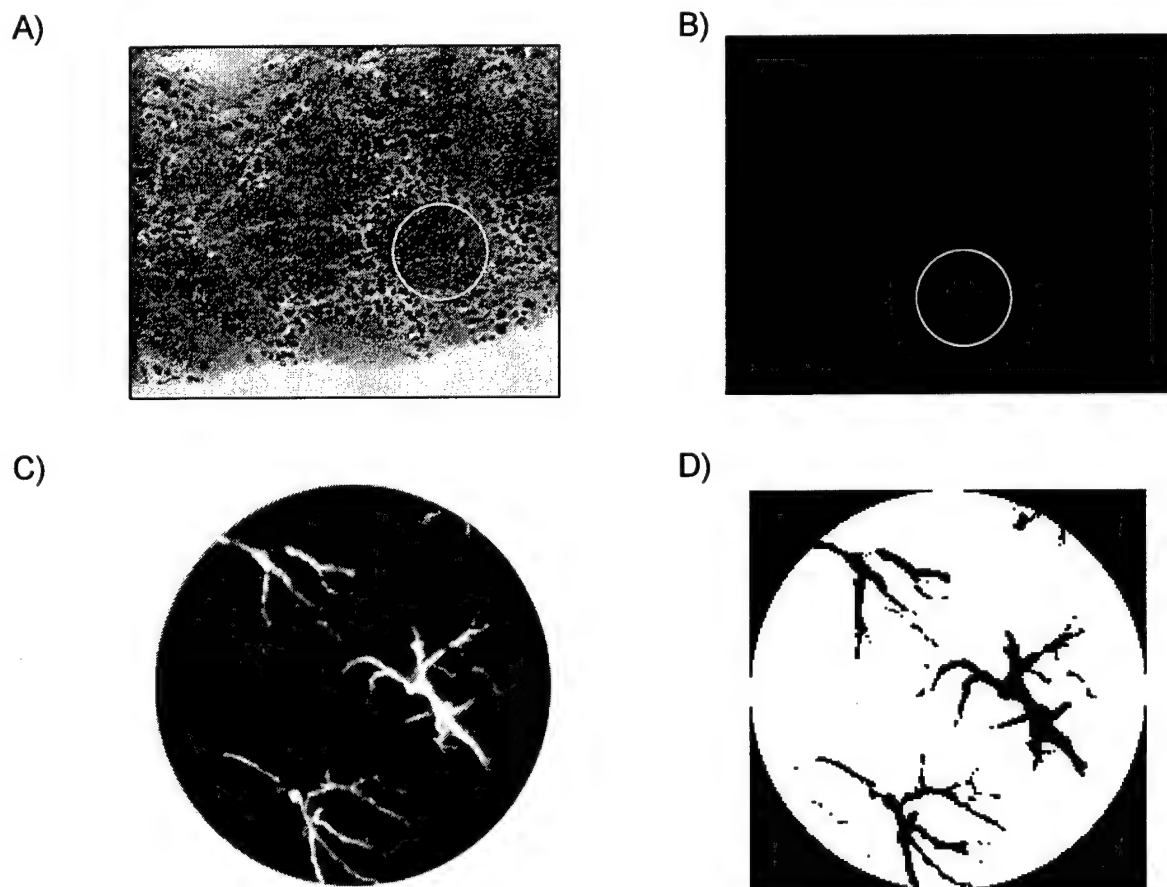


Figure 2. Schematic representation of densitometric analysis of GFAP immunoreactivity. Glomeruli are localized in hematoxylin and eosin stained section (A). Electrofluorescent image of GFAP stained adjacent section of olfactory bulb containing the same glomeruli is photographed (B). Three glomeruli are selected and a circular image of each GFAP stained glomeruli is cut out and exported to ImageTool software where images were converted from grayscale (C) to true black and white (D). Density of black in each image is measured and averaged for each structure in each section as a representation of GFAP staining intensity. Results from the three glomeruli in each section are averaged to give the final measurement for the animal.

Statistical analysis of data

GFAP measurements were percentages of fields that showed fluorescence so an arcsine square root transformation was applied before analyses. Mixed model analysis of variance was used to test whether GFAP immunoreactivity varied by type of exposure, days post exposure, and by sex. A separate analysis was performed to assess whether endotoxin affected GFAP immunoreactivity. Staining batch was included in the model as a random effect. Mixed model analyses were performed using SAS v.8.2, Proc Mixed. Model parameters were estimated by maximum likelihood and Satterthwaite degrees of freedom were used to calculate F-ratios. Generalized least-squared adjusted means were obtained and back-transformed for reporting trends in GFAP immunoreactivity that are adjusted for other effects. Residuals from analyses were collected and examined for normality and for heterogeneous variance. If the arcsine transformation failed to yield an analysis with homogeneous and normally-distributed errors then a rank-transformation was applied to the original data prior to analyses.

Results Task 3: Neuroinflammation in brains from short-term exposures

Effect of exposure

Intensity of GFAP immunoreactivity was first analyzed for groups not previously exposed to endotoxin. Residuals were normally distributed ($P > 0.15$), but the size of residual errors increased with the size of the predicted values. Analyses were therefore performed on ranks.

GFAP immunoreactivity was significantly affected by type of exposure, time after exposure, and by the interactions day*sex and exposure*day*sex (Table 7). At the initial sacrifice timepoint, 2 h after exposure, for both genders combined, glomeruli from uranium or tantalum exposed brains had significantly higher GFAP immunoreactivity than the comparable air controls (UO_3 , $p < .001$; UO_2 $p < .008$; $\text{UO}_2 + \text{UO}_3$ $p < .04$; DUOx , $p < .002$; TaO_2 , $p < 0.007$) (Figure 3). Although all exposures resulted in elevated inflammatory responses, the soluble UO_3 showed the strongest effect, with not only the highest mean immunoreactivity, but no overlap in the 95% confidence intervals between that group and the air-exposed group. At 30 days post-exposure, animals from all groups showed GFAP immunoreactivity similar to what was observed in exposed animals at 2-hr post-exposure.

Male and female animals differed in response, with exposed male animals showing a greater GFAP immunoreactivity at the 0-day sacrifice point, but generally a lower GFAP immunoreactivity at the 30 day sacrifice point.

Table 7. Fixed effects summary of mixed model analysis of factors affecting GFAP activity in rat glomeruli.

Effect	Numerator DF	Denominator DF	F value	Pr > F
Exposure Type	5	63.2	3.97	0.003
Day	1	67.3	2.00	0.162
Exposure*Day	5	64.5	2.02	0.087
Sex	1	62.7	0.01	0.935
Exposure*Sex	5	63.3	0.91	0.481
Day*Sex	1	68.8	13.77	<0.001
Exposure*Day*Sex	4	63.1	3.05	0.023

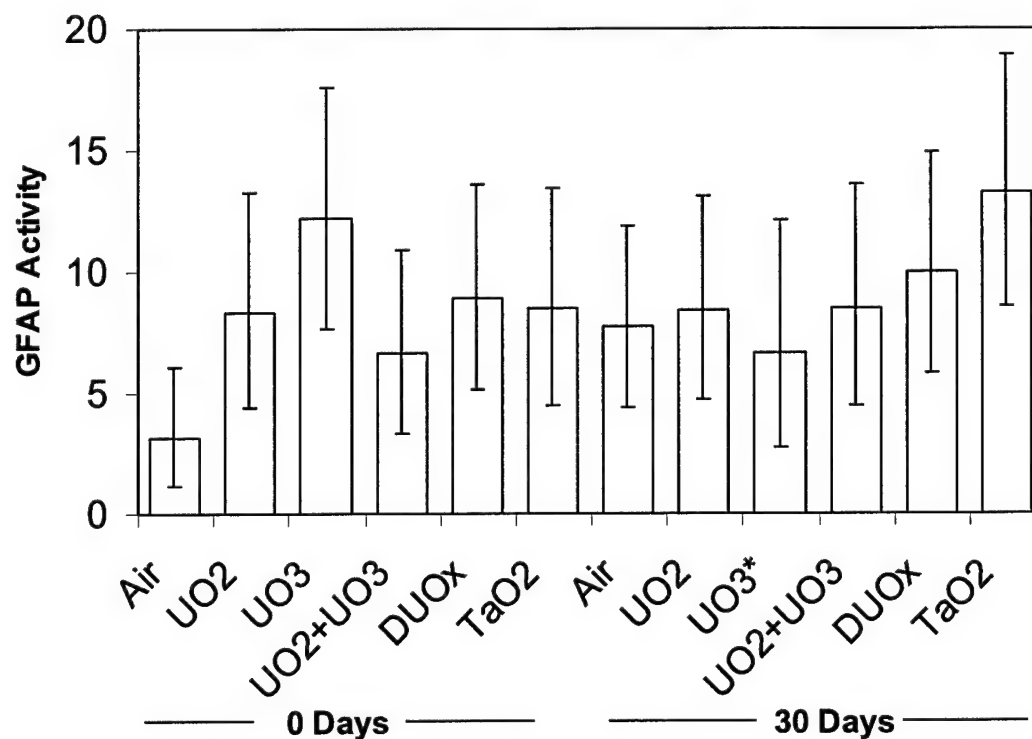


Figure 3. GFAP activity in rat glomeruli from rats exposed to uranium oxides or depleted uranium oxide for 15 min. Tissue was analyzed immediately after (0 days) or 30 days post-exposure. Bars are generalized least squares estimates and error bars are 95% confidence intervals. The 30 day UO₃* estimate was based on males only due to early death of female animals.

Effect of endotoxin

Endotoxin produced an overall increase in GFAP activity, but the response was modified by interactions with exposure type and sex (Table 8). Generally, GFAP immunoreactivity was greater in endotoxin pretreated animals regardless of exposure condition or survival time. The exception to this was the DUOX exposed group where at 0 days the endotoxin-treated group showed less immunoreactivity (Figure 4). Generally, gender was a complicating factor in interpreting the effects of endotoxin (Figure 5). Because of the limited number of animals for each gender, the gender effects are difficult to interpret.

Table 8. Fixed effects summary of mixed model analysis of endotoxin effects on GFAP activity in glomeruli.

Effect	Numerator df	Denominator df	F Value	Pr > F
Endotoxin	1	65.6	5.50	0.022
Exposure Type	2	66.0	1.06	0.353
Exposure*Endotoxin	2	65.0	3.36	0.041
Day	1	73.1	9.67	0.003
Day*Endotoxin	1	73.6	1.44	0.234
Exposure*Day	2	70.4	0.70	0.502
Exposure*Day*Endotoxin	2	68.2	2.29	0.109
Sex	1	66.3	0.13	0.720
Sex*Endotoxin	1	65.2	0.03	0.867
Exposure*Sex	2	66.4	2.72	0.073
Exposure*Sex*Endotoxin	2	66.3	3.86	0.026
Day*Sex	1	72.9	2.22	0.140
Day*Sex*Endotoxin	1	66.5	0.68	0.413
Exposure*Day*Sex	2	66.3	0.81	0.449

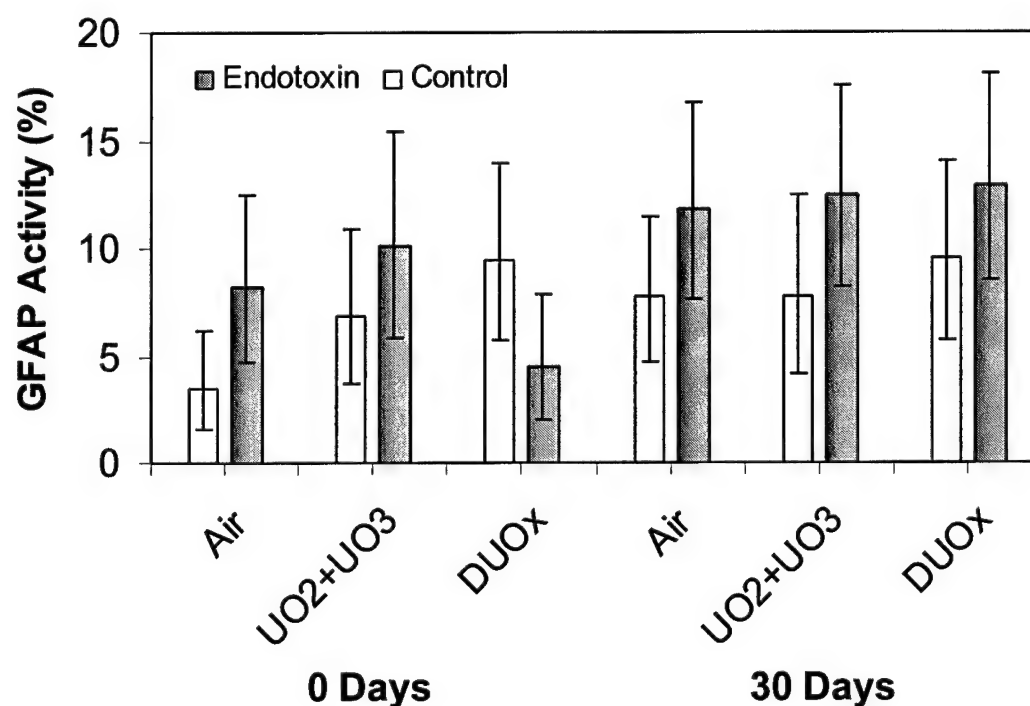


Figure 4. GFAP activity in glomeruli from rats with or without endotoxin-induced inflammation of the nasal mucosa prior to 15 min uranium oxides or depleted uranium oxide exposure. Tissue was analyzed immediately after (0 days) or 30 days post-exposure. Bars are generalized least squares estimates and error bars are 95% confidence intervals.

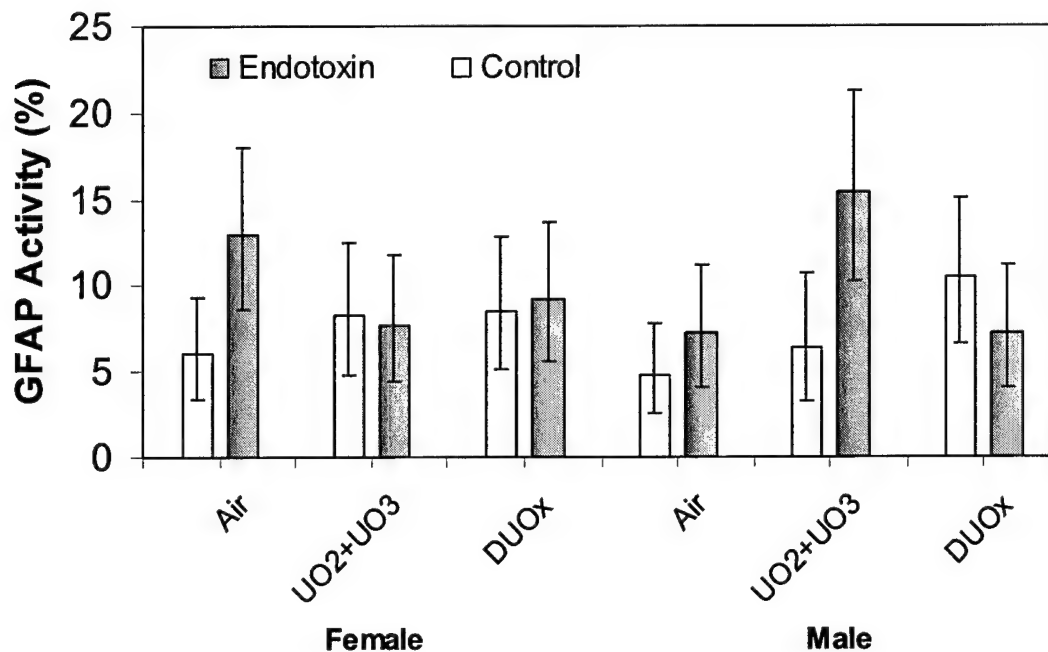


Figure 5. Exposure type, sex, and endotoxin effects on GFAP activity in rat glomeruli. Bars are generalized least squares estimates and error bars are 95% confidence intervals.

TASK 4: *One kidney and one lung from each sacrificed animal will be analyzed by AAS for inhaled metal content.*

Methods Task 4: Uranium content in kidney from short-term exposures

Technical issues: Security issues related to 9-11 resulted in unpredictable restrictions on the shipping of radioactive materials, which affected our ability to transport tissues between Albuquerque (Lovelace Respiratory Research Institute[LRRI] or UNM) and Lawrence Livermore National Laboratory (LLNL). Additional time delays occurred due to protocol development for shipping uranium exposed tissues from Albuquerque to LLNL for analysis. Digestion and ashing protocols were assessed. It was determined that the ashing resulted in higher data quality. Therefore, final protocols required weighing and lyophilizing tissues at Lovelace Respiratory Research Institute, shipping lyophilized tissue to LLNL during lower security alert states, and subsequently ashing at LLNL.

At the same time new analytical procedures at LLNL allowed us to utilize more robust analyses than were originally planned. Rather than analysis by Atomic Absorption Spectrophotometry, we are now analyzing by Inductively coupled, plasma atomic emission spectrometry (ICP-AES).

One kidney from each animal was prepared for analysis by dry ashing at 550°C. To ensure confidence in the analytical accuracy of the data, tissues are ashed for 48 hours. The ashed samples are digested for time periods of 1-2 days in 6 ml of nitric acid and metal content was

analyzed using ICP-AES. Analysis of standard samples with known uranium content revealed an of 0.3 µg/g kidney.

Results Task 4: Uranium content in kidney from short-term exposures

To date kidneys from 15 animals sacrificed 30 days after short-term exposure to UO₂, UO₃, UO₂ + UO₃ or DUOx have been analyzed. The uranium levels were below the detectable level for all samples. Analysis of additional samples is ongoing. These data and preliminary data from 0 day sacrifice point are consistent in showing no detectable uranium in the kidneys following the 15 minute exposures. Currently kidney tissue from those UO₃ exposed female animals that died between the 0 and 30 day sacrifice times is in progress. These animals showed significant lesions in the kidneys to be the cause of death, and therefore, the concentration of uranium in the kidneys becomes an important factor in understanding toxic responses in the kidney.

TASK 5: *Longer duration exposures will be performed in year 2 and 3. Serial sacrifices of rats exposed via nose-only inhalation for 8 hours to aerosols of a mixture containing by weight 50% UO₂ and 50% UO₃ at a dose of 1 mg/m³. Thirty animals will be exposed in each group. A separate air-only exposed control group will be employed as vehicle control. For each group rats will be sacrificed in sets of 6 per group immediately following exposure, and at 30, 180 and 360 days post-exposure. Tissue analyses will begin in Year 2, but substantially carry over to Year 3.*

Methods Task 5: Longer-term uranium aerosol exposures

Animals

A total of 146 (73 male and 73 female) Fischer 344 rats, 9-10 weeks old, (Harlan Sprague Dawley, Indianapolis, IN) were used. All rats were quarantined for 10 days, housed 2 to 3 per cage in shoebox cages with hardwood chip bedding. They were fed Teklad Certified Rodent Diet (W). Food and water were available *ad libitum* except during exposure. The animal rooms were maintained at 20-22°C and 30-70 % relative humidity. A 12-hour, light/dark cycle, was maintained with lights on at 0600. The rats were randomized by weight into exposure groups of 20 or 30 rats each, equal numbers of males and females (Table 9). Rats were identified by tail tattoo using an alpha numeric numbering system.

Endotoxin Instillation

To induce inflammation in the nasal mucosa, two groups of rats (Table 9) were intranasally instilled with endotoxin (Sigma Chemical Co., St. Louis, MO, Lipopolysaccharide from *Pseudomonas aeruginosa* Serotype 10, 1 mg/ml) following the procedures of Harkema and Hotchkiss (1991). Forty-eight hours before exposure to particulate aerosols or air, rats were

anesthetized by halothane inhalation, removed in a light plane of anesthesia, and instilled with endotoxin (200 µg total) by placing 2 - 50 µl drops in each nostril.

Generation of UO₂ + UO₃ aerosols

Uranium Oxides were purchased from CERAC, Inc. P.O. Box 1178, Milwaukee, WI 53201-1178 (UO₂ – CAS# 1344-57-6 50 mesh, 99.8% purity and UO₃ – CAS# 1344-58-7 Powder, 99.8% purity). Aerosol of the UO₂ + UO₃ mixture was generated using a Venturi powder disperser (Cheng et al., 1989) and a screwfeeder (Model 100, AccuRate, Whitewater, WI). All stock materials required prior ball milling to approximately 5 mm and sieving to achieve a material size suitable for aerosol generation. Aerosols generated by the Venturi powder disperser and screwfeeder system were diluted with clean, filtered air and passed through a cyclone to remove the fraction of aerosol larger than approximately 5 microns. The aerosol was then fed into a 96-port nose-only exposure system which was operated at a flowrate of approximately 20 L/min. The screwfeeder speed was adjusted to deliver the target concentration to the exposure chamber. These procedures follow Standard Operating Procedures used within LRRRI (Dunnick et al., 1988; Raabe et al., 1973). Prior to beginning actual exposures, all aerosols were tested in the exposure system to ensure target concentrations were achievable, could be reliably generated, and maintained for the necessary duration. Also, aerosols were evaluated for particle size and chemical purity.

Inhalation exposures and animal sacrifices

Before exposure, rats were conditioned to nose-only restraint tubes for at least two periods, the first for about 20 minutes and the second, conducted on a separate day, will for about 40 minutes. Rats with or without prior endotoxin instillation were exposed nose-only for 1 day (6 h) or 30 days (6 h/day, 5 days/week for 6 weeks) to 1.0 mg/m³ of UO₂ + UO₃ mixture or to air only (control group).

Animal sacrifices and tissue collection

Some rats were sacrificed at day of completed exposure (within 2 h – 0 day sacrifice) by CO₂ inhalation and exsanguination by cardiac saline perfusion. The brain was removed and frozen in liquid-nitrogen-cooled isopentane at –36°C and transferred at the end of the day to –80°C for long term storage. Spinal cords were removed and fixed in 4% paraformaldehyde.

The nose, with skin and lower jaw removed was fixed in 4% paraformaldehyde. The left and right lungs were weighed. The left lung was perfused with 4% paraformaldehyde and the right was frozen. The larynx, trachea and bronchial lymph node were fixed in 4% paraformaldehyde. The left and right kidneys were weighed. The left kidney was fixed in 4% paraformaldehyde and the right was frozen. Both femurs were weighed and frozen for chemical analysis.

Table 9. Number of animals in 1 or 30 day exposures

EXPOSURE		NUMBER OF ANIMALS PER TIMEPOINT AFTER EXPOSURE				
Material	Time	0 days	30 days	180 days	360 days	Spare Rats
Air	30d	2m/2f	2m/2f	2m/2f	2m/2f	2m/2f
UO ₂ +UO ₃	30d	3m/3f	3m/3f	3m/3f	3m/3f	3m/3f
UO ₂ +UO ₃	1d	3m/3f	3m/3f	3m/3f	3m/3f	3m/3f
UO ₂ +UO ₃ + Endotoxin	1d	3m/3f	3m/3f	3m/3f	3m/3f	3m/3f
Endotoxin + air	1d	3m/3f	3m/3f	3m/3f	3m/3f	3m/3f
Air	1d	2m/2f	-	-	-	1m/1f
Total number of rats		32	28	28	28	30

Table 10. Sacrifice dates for 1 or 30 day exposures

EXPOSURE		DATE OF SACRIFICE AFTER END OF EXPOSURE			
Material	Time	0 days*	30 days	180 days	360 days
Air	30d	10/6/03	11/6/03	4/6/04	10/6/04
UO ₂ +UO ₃	30d	10/6/03	11/6/03	4/6/04	10/6/04
UO ₂ +UO ₃	1d	10/1/03	11/1/03	3/30/04	10/1/04
UO ₂ +UO ₃ + Endotoxin	1d	10/1/03	11/1/03	3/30/04	10/1/04
Endotoxin + air	1d	10/1/03	11/1/03	3/30/04	10/1/04
Air	1d	9/8/03	-	-	-

* 0 day sacrifices completed

Results Task 5: Long-term uranium aerosol exposures

Exposure Atmosphere Characterization

The UO₂ + UO₃ concentration in the aerosol was 1.02 ± 0.12 mg/m³, size distribution was 1.66 ± 0.01 μ m with a sigma-g of 1.55 ± 0.11 . Target concentration was 1 mg/m³. PIXE analysis of filters from the incubation chambers confirmed only uranium to be present in each filter. Minimum detection limits for other elements were ~ 1000 mg/kg.

Exposure and sacrifices

Longer term animal exposures have been completed during the weeks of Aug 25th – October 6th. Zero day exposures were completed on October 1st. In addition to the 1 day exposure, we have performed a series of 30 days exposures originally within Year 3 scope of tasks. Table 9 outlines the number of animals per exposure group.

Sacrifices 2 hr after (0 day sacrifices) the 1 and 30 day exposures have been performed during the first half of October 2003. Additional sacrifices and tissue analysis will be performed predominately in Year 3 but for the later time-points be extended in to Year 4. Dates of performed and planned sacrifices are presented in Table 10.

WORK BEYOND IDENTIFIED SCOPE OF PROPOSAL

Recently published work (Haley 2003, Horner et al. 2003, Rose 2003) suggests that Gulf War veterans have an increased risk of developing amyotrophic lateral sclerosis (ALS), a neurodegenerative disorder affecting upper and lower motor neurons (Cleveland and Rothstein 2001). We have therefore collected the spinal cords from the 1 and 30 days exposed animals in order to evaluate the influence of uranium aerosol exposure on motor neuron death and neuroinflammation in the spinal cord.

Spinal cords were fixed for 2 days in 4% paraformaldehyde and transferred to 10% sucrose solution for cryoprotection. The cervical and lumbar enlargements of the spinal cord will be cryosectioned at 10 μ m thickness and immunohistochemically evaluated for loss of large motor neurons, identified as Choline Acetyl Transferase-immunoreactive neurons larger than 25 μ m in diameter (Klivieny et al. 1999). In addition, since there is evidence for neuroinflammation as a contributor to ALS pathogenesis (Hall et al. 1998, Almer et al. 2001, Malaspina et al. 2001, Drachman et al. 2002) the spinal cords will also be evaluated for immunoreactivity to GFAP to identify astrocytes and MAC-1 to identify microglia. If uranium exposure leads to neurodegeneration or inflammation in spinal cords, more detailed mechanisms of cell damage, e.g. mitochondrial status and apoptosis will be evaluated. Dr Karlsson, post-doctoral fellow in Dr Lewis laboratory, has previously been performing similar histological analysis in a transgenic mouse model of ALS during a post-doctoral period at University of Hawaii (Karlsson et al. 2002). The tissue analysis will begin in Year 3.

During Year 1, depleted uranium exposures were included that were beyond the scope of the original proposal. In developing the initial proposal, difficulties in obtaining depleted uranium suitable for aerosol exposures caused us to use surrogates varying in solubility rather than including depleted uranium. Because we were able to obtain depleted uranium oxide during Year 1, however, we did include those exposures for comparison. Results for neuroinflammation following the high concentration, short-term depleted uranium exposures are presented in Figures 3 and 4, and suggest that the $UO_2 + UO_3$ surrogate may underrepresent the response to $DUOx$ somewhat. Because the costs of including DU in the remaining exposures in terms of additional animals and maintenance, the decision was made to not continue including $DUOx$ groups in subsequent exposures. However, we do still have the source material and the protocols for generating those aerosols and running those exposures are in place and could be continued at a later date to clarify uncertainties in the data.

KEY RESEARCH ACCOMPLISHMENTS DURING YEAR 2

- Completion of sacrifices for 15 min short term exposures initialized in Year 1.
- Full analysis of uranium content in several brain regions from short-term exposed animals sacrificed 2 h (0 day) and 30 days post-exposure.
- Analysis of uranium content in kidneys from short-term exposures has begun.
- Extended evaluation of GFAP immunoreactivity in olfactory bulb glomeruli from short-term exposed animals sacrificed 2 h (0 day) and 30 days after exposure.
- Completion of longer-term exposures (1 day and 30 days) to $0.1 \text{ mg/m}^3 \text{ UO}_2 + \text{UO}_3$
- Sacrifice of the first group of animals (0 days) from longer-term exposures mentioned above.

REPORTABLE OUTCOMES

Data from the short-term uranium exposures have resulted in an abstract submitted to "Society of Toxicology Annual Meeting". The poster is to be presented in Baltimore in March 2004. The abstract is attached as Appendix 1.

CONCLUSIONS

All animals subjected to short-term exposures of uranium, depleted uranium, and tantalum oxides have now been sacrificed. Tissue analysis during Year 2 has mainly been focused on evaluation of brains, but analysis of metal content in kidneys is currently ongoing.

There was no detectable uptake of uranium in any of the investigated brain structures after 15 min short-term exposure in brains from animals sacrificed the same day as exposures or 30 days thereafter. The particle size was small enough for deposition (see Table 3). The inflammation observed suggested that soluble forms of uranium were affecting the CNS even with this short-term high-dose exposure, however, and make the continued analysis of the longer-survival animals still of interest. These results should be completed within the next 6-months now that our cryostat is again functioning. Although the short exposure duration did not result in observable uptake in all animals, two animals did show indications of entry at 30 days post exposure. Preparation of nasal tissue is in progress to determine whether or not any differences in structure, inflammatory status, or other proteins affecting transport may provide clues as to whether or not this observation is valid. It is consistent with our thinking of the nose-brain barrier that individual rather than group effects may be of more interest with respect to toxicity. Especially when exposure durations are short, transient fluctuations in properties of the olfactory mucosa and related barrier may account for differences across individuals within an exposure category, modeling the real-world situation where only some exposed individuals are affected. We hope to address this issue further in our ongoing 1 day and 30 day exposures to the same concentrations of uranium.

Evaluation of GFAP immunoreactivity in olfactory glomeruli strongly suggests that inhalation of uranium oxides, even at these short exposure durations, and to some extent also tantalum oxide, can induce a neuroinflammatory response in the brain. Solubility of the material

determined the degree of inflammation observed, with UO_3 showing the greatest degree of inflammation. The increase in GFAP immunoreactivity at 30 days post-exposure occurred in all groups including the animals exposed only to clean, filtered air. These data suggest that response was related to some characteristic in the animal's housing environment rather than the initial exposure conditions. Again, the longer term exposures to the same concentrations of uranium oxides for durations up to 30 days should help to clarify these results. Induction of nasal inflammation by endotoxin instillation in the nostrils prior to uranium exposures also increased neuroinflammation in glomeruli, and this effect did appear to interact with that of the exposure to the uranium oxides, although the interaction was not pronounced. Again, following up on this finding with longer-duration exposures seems warranted.

The gender differences observed here are not readily interpretable due to the inconsistency of the results and the limited number of animals. Both genders were included in the exposure groups and the determination to analyze by gender separately was intended as a pilot analysis to determine whether gender differences should be followed-up in a design with a greater number of animals to clarify findings. It is interesting to note, however, that significant interactions between gender and exposure conditions were observed in both the endotoxin and non-endotoxin conditions. The finding that the 0 day sacrifice animals showed a greater inflammatory response in uranium-exposed males than females, with females showing a greater magnitude of response at 30 days may lend credence to the hypothesis noted above that the 30 day inflammatory responses were related to something other than the initial exposures. Again, comparing the 1 and 30 day exposures to the same concentrations at these 30 day and longer sacrifice times will clarify the gender interactions as well.

The limited data obtained so far does not show any significant uranium deposition in kidneys from short-term exposed animals at 0 or 30 days post-exposure. However, during Year 1 we reported early deaths (within 2 weeks after exposure) in primarily female animals exposed to UO_3 for 15 min. Females were more sensitive than males, and necropsy data revealed acute tubular necrosis and uremic pneumonia, consistent with uranium toxicity. Nevertheless, data obtained so far does not show any significant uranium deposition in kidneys from short-term exposed animals. Similarly, UO_3 caused neuroinflammation although no UO_3 deposition could be detected in brains from the same animals. Analysis of the kidneys from these early death animals is in progress. The concentration of uranium in the kidneys of those animals will be important for our understanding of target organ concentrations associated with damage to the tissue, especially following these acute, high dose exposures.

Additional immunohistochemical evaluation of neurodegeneration and neuroinflammation in brains from short-term exposures is currently ongoing. We estimate that we are approximately 1-2 months behind schedule in completing the brain tissue analysis due to breakdown and necessary time for repair of the cryostat used to section brain tissue during Year 2 as discussed in the results section. This issue has now been resolved and we have been able to make substantial progress in the last few weeks. In addition, we have an additional postdoctoral associate in our UNM laboratory and plan to hire an additional technician to facilitate these analyses.

Continued uranium oxide exposures of animals has been performed successfully. In addition to 1 day exposures planned for Year 2, we have also performed 30 day exposures that were initially planned for Year 3.

In Year 3, we should also be able to complete all remaining histopathological assessments from all exposures groups sacrificed to date.

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INHALATION OF URANIUM OXIDE: PHYSIOLOGICAL EFFECTS ON RATS

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Depleted uranium (DU) has been implicated as a potential factor contributing to Gulf War illness. Upon impact, DU-containing armor penetrators burn and release uranium dust particles of respirable size. Inhaled metals can be directly transported into the brain via the olfactory system, and inflammation in nasal epithelium may increase brain metal uptake further. We therefore investigated the deposition of uranium in brain and kidney as well as neuroinflammatory response in olfactory bulb. Rats were exposed to either: a) insoluble UO₂, b) soluble UO₃, c) 50% UO₂ + 50% UO₃, d) DU oxide (DUOx), e) TaO₂ (negative control) or f) air (control) for 15 min in a nose-only inhalation chamber. The metal concentration in aerosols ranged from 300-600 mg/m³ and particle size was 1.5-2.4 µm. Nasal inflammation was induced in a subset of animals by endotoxin instillation in both nostrils 48 hours prior to air, DUOx or UO₂ + UO₃ exposure as above. Animals were sacrificed on day of exposure (0 day) or 30 days post-exposure for tissue analysis of uranium content. Astroglial response in olfactory bulbs at the same timepoints was evaluated using glial fibrillary acidic protein (GFAP) immunoreactivity. Of the 30 rats exposed to UO₃, 12 females and 3 male rats died within 12 days after exposure and were found to have acute renal tubular necrosis and uremic pneumonia. There were no early deaths in other experimental groups. Uranium levels in kidneys as well as brain olfactory system were below the detectable level at 0 and 30 days in all experimental groups. Nevertheless, GFAP intensity in olfactory bulb glomeruli was significantly elevated in UO₃ exposed animals at 0 and 30 days compared to air-exposed control rats. There was a trend for DUOx to increase GFAP response at 0 days. Thus, uranium oxide can, even at levels too low for detection in brain, lead to neuroinflammation. Effect of uranium on other tissue types and brain regions is currently under investigation.